Differential Inhibition of Cutaneous T-Cell–Mediated Reactions and Epidermal Cell Proliferation by Cyclosporin A, FK-506, and Rapamycin

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Although cyclosporin A is a highly effective treatment for several skin disorders, particularly psoriasis, its use in dermatology appears limited due to drug-induced hypertension and nephrotoxicity. Newer, similar-acting anti–T-cell agents such as FK-506 and rapamycin may be more effective; therefore a comparison was made with cyclosporin A to assess their inhibitory action on T-cell responses and keratinocyte proliferation. Using a guinea-pig model of delayed-type hypersensitivity to dinitrofluorobenzene (DNFB), drugs were given systemically (25 mg/kg cyclosporin A, rapamycin; 2.5 mg/kg FK-506) and topically (0.02% and 2%) at the time of DNFB challenge or several hours after and were assessed with respect to erythema and the numbers of infiltrating T lymphocytes entering skin-challenge sites. FK-506, at all concentrations, significantly inhibited both T-cell infiltration and skin reddening when used by both routes. Rapamycin displayed no inhibitory effect, whereas cyclosporin A only suppressed the erythema response when given systemically. The inhibition of normal human keratinocyte growth by the drugs was assessed using a protein dye-binding assay. After 2 weeks, FK-506 had no effect, whereas cyclosporin A and rapamycin both inhibited keratinocyte growth in a dose-dependent fashion and almost equivalently in serum-containing and serum-free keratinocyte growth medium. The findings showed that *in vivo* only FK-506 suppressed T-cell involvement in sensitized animals. In contrast, it failed to have any effect on keratinocyte growth, whereas rapamycin was more potent than cyclosporin A in inhibiting their proliferation. The future benefit of these drugs in dermatology may ultimately lie in their combined use. *Key words: cyclosporin A/FK-506/rapamycin/delayed-type hypersensitivity and keratinocytes. J Invest Dermatol 102:84–88, 1994*

Systemic cyclosporin A (CyA) has proved effective in the treatment of psoriasis, a putative autoimmune disease, and other candidate dermatoses displaying similar T-lymphocyte involvement [1,2]. Yet despite this, its use is restricted to severely affected patients because of drug-induced hypertension and nephrotoxicity. This, compounded with the difficulty in producing a completely clinically effective topical CyA preparation [3–5], suggests that its future use in dermatology, at least, may remain limited. Despite these problems, the testing of other similar-acting drugs such as FK-506 (FK) and rapamycin (RPM) is warranted; they may be less toxic than CyA or display greater topical efficacy.

Both FK and RPM are hydrophobic macrolide antibiotics; however, their sub-cellular actions are distinct. In common with the cyclic undecapeptide CyA, but at concentrations 10–100 times less, FK selectively suppresses the activation of $CD4^+$ cells by inhibiting the expression of early T-cell–activating genes encoding the cytokines interleukin(IL)-2, IL-3, IL-4, tumor necrosis factor alpha (TNFα), and interferon-gamma (IFNγ) [6,7]. In contrast, RPM, which inhibits T- and B-cell activation, is a more potent inhibitor of T-cell proliferation than CyA [8,9]. Unlike FK and CyA it does not prevent cytokine synthesis, but inhibits cytokine-mediated signal transduction as demonstrated by its inhibitory action upon the proliferation of IL-2, IL-4, and IL-6 lymphokine-dependent cell lines [10,11]. Recent work suggests that RPM interferes with the intracellular incorporation of the IL-2/receptor complex, because neither the expression nor the avidity of IL-2 receptor binding sites is diminished by the drug [8].

Clinically, FK represents a potent immunosuppressive agent, particularly in liver transplantation, and may prove superior to CyA by virtue of successfully reversing acute ongoing liver rejection [12,13]. Currently its potential value in the treatment of T-cell–dependent autoimmune conditions such as uveitis, nephrotic syndrome, and also psoriasis is being evaluated [14]. In contrast, clinical trials with RPM have only recently begun following its effective suppression of experimental organ rejection and autoimmune diseases [8,15,16]. Clearly the contrasting effects in potency and subcellular action of these novel immunosuppressive agents suggest their possible usefulness in the treatment of diseases such as psoriasis, which displays both active T-cell involvement and altered keratinocyte responsiveness to growth regulatory cytokines [17,18]. This study was performed to compare the efficacy of FK, RPM, and CyA in suppressing experimental T-cell–mediated skin reactions and inhibiting the growth of cultured human epidermal cells grown in serum-free and serum-containing media.

MATERIALS AND METHODS

Drugs CyA (Sandoz Ltd, Basle, Switzerland), FK (Fujisawa Pharmaceutical Company, Osaka, Japan), and RPM (Wyeth-Ayerst Research Laboratories, Princeton, NJ) were dissolved in a vehicle of 10% ethanol (CyA, RPM) or methanol (FK) and olive oil (BDH, Poole, Dorset, U.K.) for *in vivo* studies. For tissue culture the drugs were dissolved in alcohol that was

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Abbreviations: CyA, cyclosporin A; FK, FK-506; KGM, keratinocyte growth medium; RPM, rapamycin; SFM, serum-free medium.

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diluted 1000 times by culture medium. Alcohol-containing control media contained 0.1% ethanol.

Experimental Delayed-type Hypersensitivity Reactions (DTH) Dunkin-Hartley guinea pigs purchased from David Hall, Newchurch, Staffs, U.K., weighing 800–1000 g, were sensitized to 1-fluro-2, 4-dinitrobenzene (DNFB) by epicutaneous application of 50 μl 10% (w/v) DNFB solution in acetone : olive oil (1 : 1) on the dorsum of one ear. Animals were challenged 8 d later by application of a non-irritant dose of 20 μl DNFB solution (0.5% w/v) in acetone : olive oil (4 : 1) to the shaved flank and received drugs (25 mg/kg body weight, CyA and RPM; 2.5 mg/kg FK) or vehicle (1 ml/kg) systemically by gavage immediately (0 h) or 5 h later (+5 h). Animals were treated again with drugs 24 h later. Guinea pigs receiving topical drugs were challenged as before with DNFB, and immediately after drying (0 h) they received one application to the test site of topical drugs (20 μl of 0.02% or 2% w/v) or administration was delayed for 6 h (+6 h). Animals received another application of drugs 6 h later.

Assessment of DTH The erythematous reactions in guinea pigs, which were given topical and systemic drugs, were assessed "blind" at 24 and 48 h, respectively, by two independent assessors. Test sites were scored using the following scale: 0, no change; 0.5, feeble reaction; 1, patchy pink spots; 2, confluent pink; 3, red but not elevated; 4, red and elevated; 5, red, elevated, and ulcerating.

To assess the extent of inflammatory T-cell infiltration, skin biopsies were excised from the center of the reaction sites, snap-frozen, and cryostat sections (6 μm) were fixed for 20 min in acetone prior to staining. The three-stage alkaline phosphatase–anti-alkaline phosphatase method was used to demonstrate the pan T-cell marker on guinea pig T lymphocytes by using the primary mouse monoclonal antibody CT7 (Dr. R. J. Scheper, Pathological Institute, Free University Hospital, Amsterdam, the Netherlands) diluted 1 : 200 in tris-buffered saline (TBS). Secondary antibody, rabbit anti-mouse Igs (Dako, Copenhagen, Denmark) was diluted 1 : 20 in TBS containing 10% normal guinea pig serum. An assessment of cellular infiltration was performed on coded sections by counting positively stained cells in the epidermis and upper papillary dermis using a point count method with an eyepiece grid.

Preparation of Keratinocytes Normal human pediatric foreskins, previously washed in 10 × strength antibiotics in Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, Scotland, UK) for 30 min and soaked in 1 × antibiotic solution (penicillin, 100 u/ml; streptomycin 100 μg/ml, and including 2.5 μg/ml amphotericin B; Flow Laboratories, Irvine, Scotland, UK) were cut into small pieces and incubated overnight at 4°C in 0.25% dispase (Boehringer Mannheim, Lewes, East Sussex, UK) in DMEM. The epidermis was peeled from the dermis and incubated for 30 min at 37°C in 0.125% trypsin in Ca^{++}- and Mg^{++}-free PBS containing 0.02% ethylenediamine tetraacetic acid (EDTA; Flow). Trypsin was inactivated by adding DMEM + 10% fetal calf serum (FCS; Gibco) and a single-cell suspension was obtained by filtering through wire gauze. After washing, keratinocytes were resuspended in either low-Ca^{++} (0.09 mM), serum-free keratinocyte medium (SFM; Gibco) supplemented with 5 ng/ml recombinant epidermal growth factor (EGF) and 50 μg/ml bovine pituitary extract, or in serum-containing keratinocyte growth medium (KGM) consisting of DMEM: Ham's F-12 (Flow) (3 : 1), 10% FCS, 1% antibiotic solution, and the following growth factors; 24.3 μg/ml adenine, 5 μg/ml insulin, 0.4 μg/ml hydrocortisone, 10^{-10} M cholera toxin, and 5 μg/ml transferrin (all Sigma).

Keratinocyte Culture Primary keratinocyte cultures were established by seeding 50 μl of cells in 96-well plastic plates (Cel-Cult, Bibby Sterilin Ltd., Stone, Staffs, UK) at a density of 1×10^6/ml. Drugs (50 μl), made up in either SFM or KGM, were added at the same time as the cells (day 0). The final concentration of drugs was 3, 6, and 10 μg/ml for CyA and RPM, and 10, 100, and 1000 ng/ml for FK. Controls included cells grown in medium alone and in the drug vehicle of 0.1% ethanol in medium. On Day 3, and every 3 d thereafter, medium was replaced with drugs and fresh KGM containing EGF (10 ng/ml) or SFM. Cultures were incubated at 37°C in a 5% CO_2 atmosphere.

Measurement of Cell Growth On Days 7 and 14 plates were stained by the Coomassie Blue protein staining method [19] and the optical density (OD) of the stained wells used as a measure of cell growth. Plates were read on a Dynatech MR5000 microplate reader at 630 nm wavelength. Six wells were used for each treatment to give an average OD value.

Statistics Statistically significant differences between drug treatments and controls were assessed by the Mann-Whitney test for erythema scores and the Student t test for T-cell infiltrates and keratinocyte growth.

RESULTS

Effect of Drugs on DTH Reactions Results of the inhibition of DTH responses in guinea pigs given systemic CyA, FK, and RPM are shown in Table I. Guinea pigs given either CyA or FK at the time of (0 h) or 5 h after (+5 h) challenge with DNFB displayed significantly suppressed erythema reactions in comparison with vehicle-treated animals. Although the intensity of erythema was reduced in animals receiving systemic RPM this failed to be significant at either time. When biopsies from test sites were examined, only guinea pigs that received FK at the time of DNFB challenge had significantly fewer infiltrating T lymphocytes compared with either vehicle-treated animals or those in which FK was delayed for 5 h (+5 h).

The effects of topical application of CsA, FK, and RPM to guinea pigs undergoing DTH reactions are shown in Table II. Although the intensity of erythema in topical vehicle-treated animals was half that of animals given systemic vehicle (Table I), the inflammatory T-cell counts were similar, suggesting an unlikely interference of the vehicle with absorption of the recently applied DNFB. More probably, the vehicle produced a direct reduction in epidermal inflammation, a feature described for several bland emollients [20].

Guinea pigs receiving topical 0.2% and 2% FK at the time of DNFB challenge (0 h) showed a significant reduction in the intensity of skin reddening when compared with their vehicle, whereas only at the higher dose (2%) was there a significant difference in erythema between skin sites that received drug at either 0 or 6 h. Only those animals given FK topically (0.2% and 2%) at the time of DNFB challenge showed a significant reduction in the T-cell inflammatory infiltrate. Neither CsA nor RPM displayed any immunosuppressive effects when applied topically at 0.2 and 2%.

Inhibition of KC Growth by Drugs The effects of CsA, RPM, and FK on keratinocyte growth *in vitro* are shown in Fig 1. The addition of ethanol (0.1%) to cultures had no effect on their growth in either serum-containing KGM or SFM. Both CyA and RPM significantly inhibited keratinocyte growth at Days 7 and 14 in both media with a dose-response effect being more evident in SFM. At each time, in SFM and KGM, 10 μg/ml RPM produced almost the same degree of inhibition of keratinocyte growth as the equivalent concentration of CyA; however, at 6 and 3 μg/ml, RPM was more inhibitory than the same concentrations of CsA, although not significantly. FK demonstrated no inhibitory effect on keratinocyte growth in either KGM or SFM, apart from one concentration (100 ng/ml) in SFM.

The percentage inhibition of keratinocyte growth in KGM and SFM for each drug concentration was compared between Days 7 and 14. In KGM there was no further reduction in cell growth at Day 14 compared with Day 7 for any drugs. In contrast, in SFM keratinocytes grown in the presence of CyA showed a further average reduction in growth at Day 14 compared with Day 7 of 60%, which was significant ($p < 0.05$) at 6 μg/ml CsA. By Day 14 RPM had produced a further 70% reduction in growth compared with Day 7 values, which were significant at 3, 6 ($p < 0.01$), and 10 μg/ml ($p < 0.001$).

Keratinocytes grown in medium, the ethanol vehicle, and FK had formed an undifferentiated monolayer by Day 14. In comparison, 10 μg/ml CyA and RPM were particularly toxic, as remnants of dead cells were most evident by 7 d. Lower concentrations of CyA and RPM appeared to have a cytostatic action and produced gross abnormalities in cell appearance with 6 μg/ml CyA or RPM, and 3 μg/ml RPM.

DISCUSSION

Cyclosporin A has previously been shown to inhibit significantly DTH responses to DNFB in guinea pigs when given systemically during the initial period of T-cell sensitization [21]. In contrast, when CyA was first administered at the time of antigen challenge to murine models of hypersensitivity pneumonitis [22] and DTH to methylated BSA [23], the drug failed to prevent the accumulation of

Table I. Effect of Systemic Drugs on the DTH Response in Guinea Pig Skin at 48 h After Challenge with DNFB[a]

Drug	Dose (w/v) Given 0 + 24 h	n	Time of Drug Application after DNFB Challenge (h): Intensity of Erythema 0	Intensity of Erythema +5	Number of Infiltrating T cells/mm² 0	Number of Infiltrating T cells/mm² +5
Vehicle[b]		7	3.2 ± 0.8	3.4 ± 0.8	130 ± 32	140 ± 83
CyA	25 mg/ml	5	1.5 ± 0.9[c]	1.7 ± 0.8[d]	123 ± 43	116 ± 82
FK	2.5 mg/ml	5	1.5 ± 0.8[c]	1.7 ± 0.9[c]	32 ± 9[c,f]	84 ± 36
RPM	25 mg/ml	5	2.2 ± 0.8	2.7 ± 0.8	117 ± 47	99 ± 101

[a] Intensity of erythema was scored visually and the number of infiltrating T cells enumerated on biopsy sections. Results represent means ± SD from three experiments.
[b] Statistically significant differences between drug and vehicle for each time ([c]$p < 0.05$; [d]$p < 0.01$; [e]$p < 0.001$) and between times for each treatment ([f]$p < 0.05$) were measured using the Mann Whitney test for scores and Student t test for cell counts.

inflammatory cellular infiltrates although accompanying increases in lung volume and fluid exudates, respectively, were inhibited. Similarly, in this study, when systemic CyA (25 mg/kg) was given at the time of DNFB challenge or 5 h later, it significantly inhibited erythema, but there was no suppression of the T-cell response. Whereas systemic CyA appears to be ineffective in suppressing the participation of previously sensitized T cells, of which many will be specific skin-residing memory T cells [24], its ability to suppress the accompanying erythema suggests that these manifestations may not be entirely T cell mediated. These irritant-like features, characterized by increased vascular permeability and edema, may in fact result from the binding of haptens directly with KC, which induces their activation and release of inflammatory cytokines such as TNFα, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1, and IL-8 [25]. Evidence suggests that TNFα plays a central role in this response because anti-TNFα monoclonal antibodies totally abolished ear thickening during contact sensitivity responses in challenged mice [26]. Therefore, given that CyA can inhibit TNFα release from mononuclear leukocytes [22,27], and may also suppress KC-derived TNFα production [28], this might account for its effective suppression of erythema but not the accompanying cellular infiltrate.

In contrast to its effects when given systemically, topical CyA at 0.02 and 2% did not prevent any aspect of the DTH response in this study although previously it has been particularly inhibitory at 5% [29]. It is likely, therefore, that 2% CyA was an insufficient concentration to provide effective immunosuppression.

Unlike CyA, FK was most potent at suppressing challenge DTH reactions. Not only did it inhibit erythema when given systemically at one-tenth of the concentration used for CyA, but it also significantly suppressed the accumulation of T cells at test sites by 75% when given at the time of challenge and partially (40%), but not significantly, when delayed by 5 h. This superior immunosuppressive efficacy shown by FK has previously been demonstrated following its administration to guinea pigs with ongoing uveitis where the development of eye damage and antibodies to retinal S-antigen was suppressed [30]. Although FK may primarily inhibit sensitized or activated T cells, it also impairs alloantigen presentation by monocytes [31] and might similarly affect antigen handling by Langerhans cells.

The most important observation from this *in vivo* study was that FK was extremely effective when used topically. This agrees with recent findings by Meingassner and Stütz, who examined vascular changes in pigs undergoing DTH reactions to DNFB and found that neither CyA (10%) nor RPM (0.13 and 1.2%) suppressed skin reddening, whereas concentrations of 0.04–0.4% FK were all inhibitory [32]. In the guinea pigs even concentrations as low as 0.02% significantly suppressed erythema and, more importantly, the inflammatory infiltrate by 60%. Interestingly, RPM failed to inhibit any aspect of the DTH response when given topically or even systemically. Rapamycin does not inhibit the expression of early activation genes, as do FK and CyA, but instead doubles their transcripts in activated T cells [7]. If RPM induced similar elevations of inflammatory cytokines in hapten-stimulated keratinocytes, then this might provide the inflammatory stimulus responsible for maintaining the DTH response.

Rapamycin was particularly potent at suppressing the growth of normal human keratinocytes and exceeded that of equivalent concentrations of CyA (3–10 μg/ml), which are reportedly present in psoriatic epidermis (1–2.9 μg/ml) of systemically drug-treated patients [33]. The inhibitory action of 10 μg/ml RPM and CyA was undoubtedly due to drug toxicity, whereas lower concentrations (3 and 6 μg/ml) appeared to have a cytostatic action. Unlike CyA and RPM, FK, even at 1 μg/ml, had no inhibitory effect on cell growth. In this study the effects of each drug on the growth of keratinocytes were similar in both serum-free or -containing media; serum did not diminish the inhibitory effects of CyA or RPM.

The mechanism(s) by which RPM and CyA, but not FK, induces this growth inhibition is not known. Normal cultured keratinocytes have been reported to express reduced numbers of functional epi-

Table II. Effect of Topical Drugs on the DTH Response in Guinea Pig Skin at 24 h After Challenge with DNFB[a]

Drug	Dose (w/v) Given 0 + 6 h	n	Time of Drug Application after DNFB Challenge (h): Intensity of Erythema 0	Intensity of Erythema +6	Number of Infiltrating T cells/mm² 0	Number of Infiltrating T cells/mm² +6
Vehicle		6	1.7 ± 0.8 (n = 12)	1.3 ± 0.5	131 ± 62 (n = 12)	120 ± 58
CyA	2%	6	1.3 ± 0.4	1.9 ± 0.6	126 ± 73	108 ± 33
	0.02%	6	1.3 ± 1.0	1.2 ± 0.3	135 ± 77	145 ± 47
FK	2%	6	0.5 ± 0.6[b,d]	1.3 ± 0.4	54 ± 60[b]	63 ± 45
	0.02%	6	0.8 ± 0.8[b]	1.2 ± 0.3	52 ± 34[c]	106 ± 74
RPM	2%	6	1.0 ± 0.6	1.5 ± 0.9	98 ± 62	141 ± 75
	0.02%	6	1.3 ± 0.9	1.5 ± 0.6	145 ± 108	174 ± 80

[a] Intensity of erythema was scored visually and the number of infiltrating T cells enumerated on biopsy sections. Results represent means ± SD from two experiments.
Statistically significant differences between drug and vehicle for each time ([b]$p < 0.05$; [c]$p < 0.01$) and between times of drug administration ([d]$p < 0.05$) for each treatment were measured using the Mann-Whitney test for scores and Student t test for cell counts.

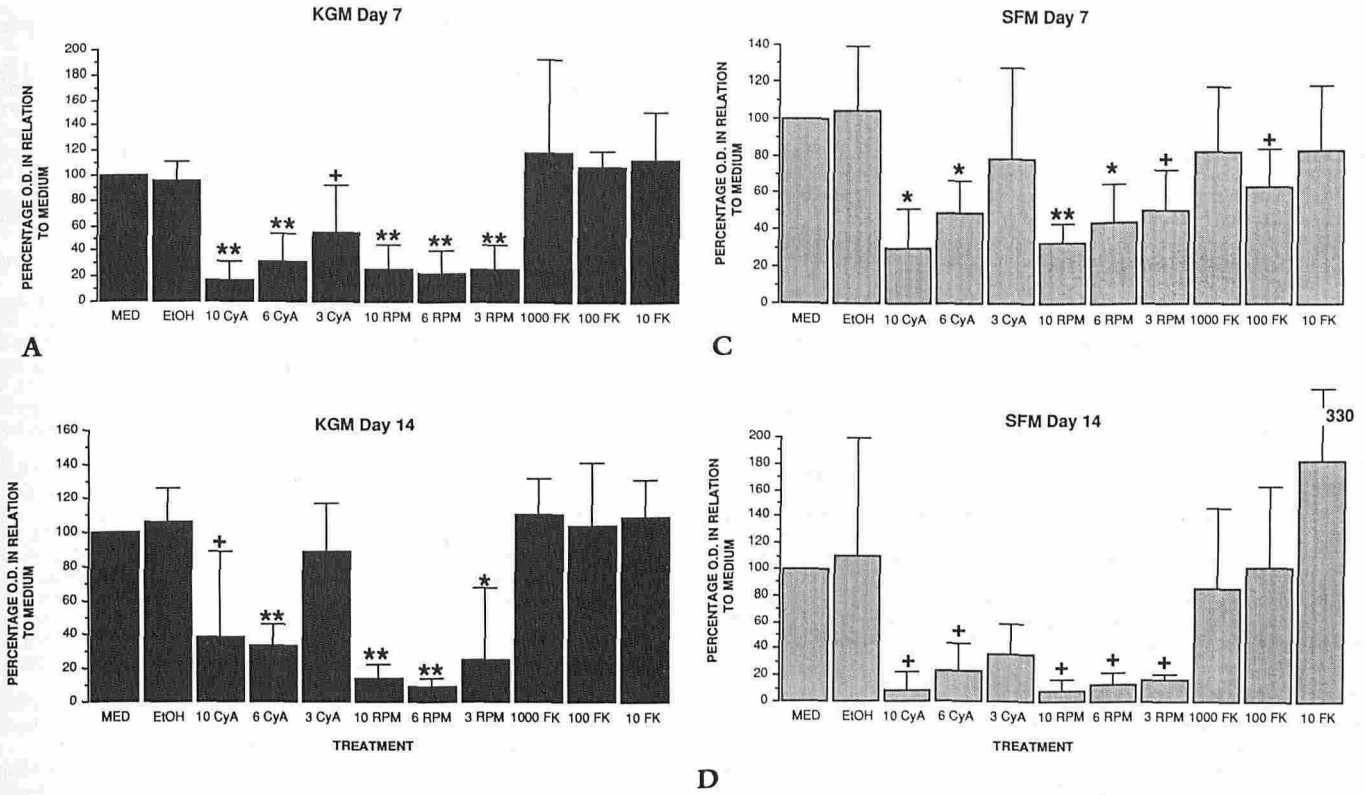

Figure 1. Drug effects on keratinocyte growth. Primary epidermal cells were grown in the presence of serum-containing medium (KGM) or serum-free medium (SFM) and drugs; medium (Med), ethanol control (EtOH), 3–10 μl/ml CyA and RPM, and 10–1000 ng/ml FK. On Days 7 and 14 cell growth in control and drug treatment groups was expressed as a percentage of cells grown only in medium. *Columns,* percentage mean ± SD from at least 5 individual *skins.* Statistically significant inhibition of cell growth by drugs was assessed by comparing drug treatments with their ethanol control; $^{**}p < 0.001$, $^{*}p < 0.01$, $^{+}p < 0.05$.

dermal growth factor receptors (EGFRs) following CyA treatment, despite producing increased amounts of its ligand-transforming growth factor alpha (TGFα) [34]. In addition, keratinocyte growth driven by basic fibroblast growth factor, another keratinocyte autocrine growth factor that mediates its effects *via* a receptor distinct from EGFR, is also affected by CyA [35]. To the same extent, RPM has also been shown to diminish the autonomous proliferation of T-cell hybridoma cells [10] and rat hepatocytes [36], where in the latter mRNA transcripts of their growth factor, TGF-beta, but not of non-growth-associated proteins such as albumin, were inhibited. It is possible that this non-immunologic inhibition of cell growth results from the blockade by CyA and RPM of their cytosolic binding proteins (BP), which may regulate cell growth in a manner similar to the FK-BP, which has recently been shown to promote hepatocyte growth *in vivo* [37].

Initial clinical assessment with FK suggests that systemic FK may be a more potent drug than CyA for the treatment of psoriasis; complete remission occurred within 4 weeks and persisted, but this was accompanied by some renal impairment [38]. As FK does not appear to affect the growth of keratinocytes, its anti-psoriatic properties possibly result primarily from an inhibitory action on T cells rather than on keratinocytes. This has been evidenced by the rapid disappearance of inflammatory infiltrates from psoriatic plaques, which well preceded any resolution of hyperkeratosis in the skin of FK-treated patients [38]. The experimental results of its use topically in this study do, however, indicate that FK either has a greater therapeutic index than CyA or better percutaneous penetration properties and, therefore, warrants fuller examination as a topical preparation. The long-term aim of producing an effective but nontoxic drug regime for treating dermatoses such as psoriasis may ultimately depend upon drug combination therapy. Recently, CyA and RPM, at doses that were individually noninhibitory have in combination acted synergistically by totally preventing rat cardiac allograft rejection [11]. This experimental approach might have some practical application to the treatment of psoriasis itself, especially because CyA is already known to be effective but at essentially nephrotoxic concentrations, and RPM displayed such potent growth-regulatory effects on keratinocytes. Although the future and more widespread use of these specific immunosuppressive drugs will ultimately be determined by their toxicity profiles, it is possible that individually CyA, FK, and RPM may provide a greater understanding of the pathologic mechanisms underlying certain dermatologic diseases.

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